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(21) International Application Number: PCT/AU92/00372 (22) International Filing Date: 23 July 1992 (23.07.92) (30) Priority data: PK 7400 24 July 1991 (24.07.91) AU (71) Applicant (for all designated States except US): UNIVERSITY PARTNERSHIPS PTY. LTD. [AU/AU]; 1st Floor, Richardson's Arcade, Armidale, NSW 2351 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): GREGG, Keith [AU/AU]; COOPER, Christopher, Lyle [AU/AU]; Institute of Biotechnology, University of New England, Armidale, NSW 2351 (AU).	(74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published With international search report.	
(54) Title: SINGLE STEP AMPLIFICATION AND SEQUENCING OF NUCLEIC ACIDS (57) Abstract Method for the amplification and sequencing of DNA or RNA. The method comprises the steps of (i) melting a double stranded nucleic acid to yield a pair of complementary nucleic acid strands, (ii) hybridising a primer to each of the strands, the primers being so chosen that the primer annealing to the sense strand is 3' to the position of the primer on the antisense strand, one of the primers being labelled so as to be capable of being visualized independently of the other primer, (iii) causing a polymerase enzyme to amplify the nucleic acid in the presence of a dideoxynucleotide analogue of one of the nucleotides present in the nucleic acid, the dideoxy analogue being present in such a concentration that a majority of the newly synthesised nucleic acid strands are terminated by incorporation of dideoxynucleotides without extending far enough to act as templates for synthesis of the opposite strand using the second primer, (iv) repeating steps (i) to (iii) sequentially a number of times, (v) repeating the steps (i) to (iv) using at least another two dideoxynucleotide analogues of the other three nucleotides present in the nucleic acid, and (vi) electrophoretically separating the reaction products of each of the repetitions of steps (i) to (iv) and visualizing the labelled strands. The other of the nucleotides of at least a part of the strand of the nucleic acid to which the labelled primer annealed between the binding sites may be determined by comparing the separated and visualized gels for each of the nucleotide analogues used.		

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- 1 -

Single Step Amplification and Sequencing of Nucleic Acids
Field of the Invention

The present invention relates to a method for the single-step amplification and sequencing of the nucleic acids deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

Background Art

It is known that DNA can be amplified by the polymerase chain reaction (PCR). During PCR double-stranded DNA, such as genomic DNA, is melted to separate the two complementary DNA chains and two primers are added one of which is complementary with a known sequence on each of the two DNA strands. In the presence of suitable nucleotides the polymerase enzyme will build up complementary strands of DNA on the primed templates until the reaction conditions are changed. The primers are so chosen that the primer annealing to the sense strand is 3' to the position of the primer on the antisense strand. Repetition of this cycle will cause the original genomic templates to be again reproduced. In addition the complementary strands formed during the first cycle will be reproduced but only to the extent of the region of the original genomic DNA which lies between and includes the two primers. The amplified DNA region between the primers is then recovered by electrophoretic purification. This PCR reaction is described more fully in Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharft, S.J., Higuchi, R. Horn, G.T. Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487-491.

It is also known to sequence DNA using a process in which a single labelled primer is annealed to suitably melted DNA. The reaction is carried out in four separate reactions, each reaction vessel containing the dideoxynucleotide analogue of one of the usual nucleotides. The presence of the dideoxy analogue in a suitable concentration causes the chain reaction to stop

- 2 -

once out of every three or four hundred times that the respective complementary nucleotide occurs in the template. The extended DNA products from each of the four reactions are run side by side on a suitable
5 electrophoretic gel and visualised. The relative positions of the bands on the gel allows the sequence of the newly synthesized template DNA to be deduced. This dideoxynucleotide sequencing reaction is more fully described in Sanger, F., Nicklin, S and Coulsen, A.R.
10 (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5483-5487.

It is also known to improve the sensitivity of sequencing reactions by thermocycling with a heat-stable DNA polymerase (see Lee, J.S. (1991) DNA and Cell Biol. 10, 87-73). In the existing proposals for using
15 polymerase amplification of DNA together with sequencing, entirely separate steps have been provided for amplification of the template DNA, purification of products and sequencing. The present invention provides for an alternative to the known processes for the
20 amplification and sequencing of nucleic acids.

Disclosure of the Invention

The present invention consists in a method for the amplification and sequencing of a nucleic acid comprising the steps of:-

- 25 i) melting a double stranded nucleic acid to yield a pair of complementary strands of nucleic acid;
- ii) hybridising a primer to each of the strands of nucleic acid, the primers being so chosen that the primer annealing to the sense strand is 3' to the
30 position of the primer on the antisense strand, one of the primers being labelled so as to be capable of being visualised independently of the other primer;
- iii) causing a polymerase enzyme to amplify the nucleic acid in the presence of a dideoxynucleotide analogue
35 of one of the nucleotides normally present in the

- 3 -

- nucleic acid, the dideoxynucleotide analogue being present in such a concentration that a majority of the newly synthesised nucleic acid strands are terminated by incorporation of dideoxynucleotides without extending far enough to act as templates for synthesis of the opposite strand using the second primer;
- 5 iv) repeating the steps i) to iii) sequentially a number of times;
- 10 v) repeating the steps i) to iv) using dideoxynucleotide analogues of at least all but one of the other nucleotides present in the nucleic acid; and
- vi) separating the reaction products of each of the repetitions of the steps i) to iv) and visualising the labelled strands to allow a determination of the sequence of at least a part of the strand of the nucleic acid to which the labelled primer annealed between the binding sites for the two primers.
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While the method according to the present invention may be carried out on RNA it is preferable to form a double stranded DNA analogue of the RNA and to sequence that. Similarly single stranded DNA may be converted to double stranded DNA and sequenced according to the present invention. The present method is particularly suitable for use with prokaryotic DNA but may also be useful in sequencing eukaryotic DNA.

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It is preferred that the reaction products of the various repetitions of steps (i) to (iv) be separated by electrophoretic separation in a suitable gel. This allows the various reaction products to be run side by side allowing a direct reading of the nucleic acid sequence from the gel. If the various repetitions are run using primers uniquely labelled, for instance by using a different fluorescent label for each repetition, it would be possible to run a single gel track and to use an

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- 4 -

autosequencer to automatically read the nucleotide sequence from the gel.

The labelled primer may be labelled in any suitable manner known in the art. One suitable manner is by
5 incorporation of a radioactive isotope of an element in the primer. Other suitable means include labelling the primer with a binding ligand such as biotin or labelling the primer with a fluorescent marker.

The distance between primers on the template can be
10 varied to alter the sensitivity. The efficiency of amplification depends upon the proportion of new DNA chains that extend far enough to act as templates for synthesis of a complementary strand using the second primer. It has been found that primer separation of about
15 250 base pairs provides reasonable levels of amplification with eukaryotic DNA and allows reliable sequence data to be obtained. In the case of prokaryotic DNA primer separation of 600 base pairs is quite possible. The method according to this invention can be run using
20 nanogram quantities of DNA however larger quantities are desirable with greater primer separation.

The results obtained are sometimes confused adjacent to the originating primer where one can get "cross banding". Closely adjacent the other primer one may also
25 find abnormalities such as "blockage". The best results are obtained from the intermediate DNA between the two primers.

It would be normal that any nucleic acid studied would include four nucleotides. It would thus be normal
30 to carry out the process of steps (i) to (iv) four times, once with the dideoxynucleotide analogue for each of the nucleotides occurring in the nucleic acid. It will be obvious that the method could be carried out for only three of the nucleotides with presence of the fourth
35 nucleotide being implied by gaps in the separation. This

- 5 -

strategy is deemed to be within the scope of this invention however it is not a preferred strategy.

Brief Description of the Drawings

Fig 1 is a diagrammatic representation of the process according to the present invention, and

Fig 2 is an electrophoretic gel showing the separation of strands of DNA for four duplicate reactions involving four different dideoxynucleotide analogues and showing the deduced nucleotide sequence of part of the DNA being investigated.

The diagram of Fig 1 shows the process of the present invention. A double stranded DNA 10 comprising a sense strand 11 and an antisense strand 12 is heated and annealed in the presence of two primers 13 and 14. Primer 13 binds to the sense strand 11 3' to the position which primer 14 binds to the antisense strand 12. This means that the synthesis on the two strands is converging in direction. The primer 14 is labelled, such as with radioactive phosphorous. This allows the strands of DNA derived from the antisense strand to be visualised at the end of the process independently of the strands of DNA derived from the sense strands. The DNA strands 11 and 12 to which the primers 13 and 14 have been annealed are divided into four aliquots 15, 16, 17 and 18.

Each of the aliquots 15, 16, 17 and 18 is amplified using a polymerase enzyme. A dideoxynucleotide analog of one of the nucleotides in the DNA is added to each aliquot. Thus dideoxycytosine triphosphosphate is added to aliquot 15 and the corresponding dideoxynucleotides based on thymine, adenine and guanine are added to aliquots 16, 17 and 18 respectively. By cycling through a process of heating and annealing each of the aliquots a large number of DNA strands of different lengths will be built up in each aliquot. In each case each of the strands 11 and 12 of the DNA will be copies in each

- 6 -

aliquot 15, 16, 17 and 18. The copying in each case will stop when a dideoxynucleotide analogue is inserted into the developing copy of the strand in place of the corresponding deoxynucleotide. By adjusting the ratio of the dideoxynucleotide and the deoxynucleotide in each aliquot it is possible to produce a majority of DNA copy strands which stop short of reaching the point at which the opposing primer will anneal to the copy strand. As an example in aliquot 15 strands 19, 21 and 22 have stopped short of reaching a length sufficient to allow binding of the primer 13. These strands will thus not be reproduced in further cycles of the heating and annealing. The strand 23, by contrast, has extended to a point where the primer 13 will anneal to it and it served as a template for the production of reverse strands of DNA.

In this way is built up four populations of strands of varying length. These populations are separated on an electrophoretic gel 24 and visualised on a suitable film. The film will only record the presence of the strands incorporating the labelled primer. The four aliquots 15, 16, 17 and 18 are run side by side on the gel 24 and the shorter strands will be carried furthest along the gel 24. Reading the gel 24 in the direction of the arrow shows that the strand sequenced reads CCTAGGGATCTA.

Figure 2 shows the visualisation of an actual gel showing the sequencing of a section of DNA from the chromosome of a rumen bacterium Prevotella ruminicola, strain AR20. The gel is read in the direction of the arrow and the section identified reads 5' to 3', GGCTTTTACAGTT.

Best Mode of Carrying out the Invention

In order that the nature of the present invention may be more clearly understood a preferred embodiment thereof will now be described by reference to the following example.

- 7 -

DNA Preparation and Sequencing Protocol

Genomic DNA for amplification sequencing can be prepared from bacterial samples by any of the standard methods (Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory). A rapid and convenient method which may be used is as follows.

1.5 ml of bacterial suspension was centrifuged at 2000 g for 5 min, resuspended in 0.5 ml of TE buffer (Tris. HCl, pH 8.0, 10 mM; Ethylenediamine tetra-acetic acid, EDTA, 1 mM) and recentrifuged at 2000 g. The bacterial pellet was suspended in 0.4 ml of TE buffer, containing 4 mg/ml Lysozyme, and incubated at 37°C for 20 min. 10 µl of 10% Sodium dodecyl sulfate (SDS) was mixed with the sample, followed by 10 µl of ribonuclease solution (10 mg/ml), and it was incubated for 20 minutes at 37°C. 10 µl of Proteinase K (10 mg/ml) was added, and the sample incubated at 55°C for 30 minutes, or until clear. The cleared solution was extracted once with an equal volume of phenol/chloroform (1:1), twice with 0.4 ml of chloroform, and the DNA was precipitated by mixing the aqueous phase with 1 ml of ethanol at room temperature for 4-5 min. and centrifuging at 12000 g for 5 min. The DNA was redissolved in 0.2 - 0.4 ml of distilled water and the purity and concentration estimated by u.v. spectrum of a 1:50 dilution.

For the four sequencing reactions, a total of approximately 5 - 10 pg of plasmid, or 5 - 10 ng of pure bacterial genomic DNA was used as template, although this quantity could be increased without adverse effects. Mixtures of bacterial DNAs were used at quantities sufficient to give at least 5 ng of target DNA in the reactions.

Four sequencing reactions were prepared, as for

- 8 -

conventional DNA sequencing with the TaqTrack kit (Promega). A single aliquot of template DNA was mixed with 20 ng each of two PCR primers, of which one had been labelled with ^{32}P . Labelling was by polynucleotide kinase reaction (Promega), with $\gamma\text{-}^{32}\text{P}\text{-ATP}$, using the manufacturer's recommended conditions, at 37°C for 80 - 90 min.

PCR reaction buffer (Promega) was used in place of the normal DNA sequencing kit buffer, and was mixed with the template and primers, and the solution adjusted to a final volume of 22 μl with distilled water. The mixture was divided, 5 μl into each of four tubes, each containing 2 μl of a specific TaqTrack dNTP/ddNTP mixture.

Reaction mixtures were overlaid with 40 μl of mineral oil, to prevent evaporation, and subjected to 25 - 30 cycles in the programmable incubator. Steps for denaturation of the double-stranded template, and primer annealing, prior to the sequencing reactions, were unnecessary.

Incubation conditions were as follows: denaturation, 92°C for 90 s; annealing, 55°C for 80 s; polymerization, 72°C for 80 s. After completion of the reaction cycles, 5 μl of formamide stop-solution was added and the tubes centrifuged to pass it through the oil layer (2000 g for 30 s). Reaction products were heated at 95°C for 3 min. Samples could be withdrawn from beneath the oil using a glass capillary tube, or the oil could be removed by two gentle ether washes.

Products were separated by loading 2 μl of each reaction mixture onto standard 6% (w/v) denaturing polyacrylamide gels containing 8 M urea in Tris-borate EDTA buffer (Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning; A Laboratory Manual. Cold Spring Harbor Laboratory). After electrophoresis, gels

- 9 -

were fixed with 12% (v/v) acetic acid for 10 minutes, washed with 1 litre of 20% (v/v) ethanol, and dried at 95°C, before being autoradiographed overnight with Fuji X-ray film.

5 During this process, a high proportion of nascent DNA chains were terminated by incorporation of dideoxynucleotides. However, in each cycle, a proportion were extended far enough to allow annealing of the opposite primer and therefore to act as templates for
10 synthesis of the opposite strand. This resulted in amplification of both strands at less than normal PCR efficiency. Concurrently, the available template was re-used at each cycle, for the sequencing reaction.

15 The time taken, to obtain sequence from a sample of chromosomal DNA, is reduced to less than half that required in conventional PCR/sequencing. Furthermore, amplification factors in this method are reduced considerably from those normally used in manufacturing template DNA. This would be expected to reduce the number
20 of errors introduced during amplification. Concurrent duplication of the reactions provides immediate sequence confirmation.

25 This technique will clearly be advantageous in processes that require detailed genetic screening of numerous DNA samples: e.g. examination of specific genes for point mutations, or in analyses that will benefit from specific strain identification. Genes in which different strains of an organism possess different DNA sequences can be analysed rapidly, using small quantities of relatively
30 impure DNA. A difference of one base-change, anywhere within the region spanned by the primers, can be detected by this method. In addition, application of the technique to RNA templates should allow rapid analysis of e.g. 16S ribosomal RNA for bacterial taxonomy, or the
35 detection and detailed identification of retroviral

- 10 -

strains.

Previous reports of thermocycling for DNA sequencing reactions have used a single, unlabelled primer, and the incorporation of radioisotopes during the reaction, to
5 label the sequencing products (Lee, J-S. (1991) DNA and Cell Biol. 10, 87-73). However, that approach is likely to result in problems through the attachment of radio-isotopes to non-target DNA, in samples that are not entirely pure. Prelabelling the primer results in
10 adequate sensitivity and avoids the complications that may arise in mixtures of DNA.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments
15 without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

- 11 -

CLAIMS:-

1. A method for the amplification and sequencing of a nucleic acid comprising the steps of:-
 - 5 i) melting a double stranded nucleic acid to yield a pair of complementary strands of nucleic acid;
 - ii) hybridising a primer to each of the strands of nucleic acid, the primers being so chosen that the primer annealing to the sense strand is 3' to the position of the primer on the antisense strand, one
10 of the primers being labelled so as to be capable of being visualised independently of the other primer;
 - iii) causing a polymerase enzyme to amplify the nucleic acid in the presence of a dideoxynucleotide analogue of one of the nucleotides normally present in the
15 nucleic acid, the dideoxynucleotide analogue being present in such a concentration that a majority of the newly synthesised nucleic acid strands are terminated by incorporation of dideoxynucleotides without extending far enough to act as templates for
20 synthesis of the opposite strand using the second primer;
 - iv) repeating the steps i) to iii) sequentially a number of times;
 - v) repeating the steps i) to iv) using dideoxynucleotide
25 analogues of each of the other nucleotides present in the nucleic acid; and
 - vi) separating the reaction products of each of the repetitions of the steps i) to iv) and visualising the labelled strands to allow a determination of the
30 sequence of at least a part of the strand of the nucleic acid to which the labelled primer annealed between the binding sites for the two primers.
2. A method as claimed in claim 1 in which the double
35 stranded nucleic acid is a double stranded DNA derived from a prokaryotic organism.

- 12 -

3. A method as claimed in claim 1 in which double stranded nucleic acid is a double stranded DNA analogue of an RNA.
4. A method as claimed in claim 1 in which the one primer
5 is labelled with a radioactive isotope.
5. A method as claimed in claim 1 in which the nucleic acid to be sequenced is derived from an eukaryotic organism and the binding sites of the primers are separated by about 250 base pairs.
- 10 6. A method as claimed in claim 1 in which the nucleic acid to be sequenced is derived from a prokaryotic organism and the binding sites of the primers are separated by at least 600 base pairs.
7. A method as claimed in claim 1 in which the reaction
15 products are separated electrophoretically.
8. A method as claimed in claim 7 in which the reaction products of the replications of the steps (i) to (iv) are run side by side on a single eletrophoretic gel.

1/2

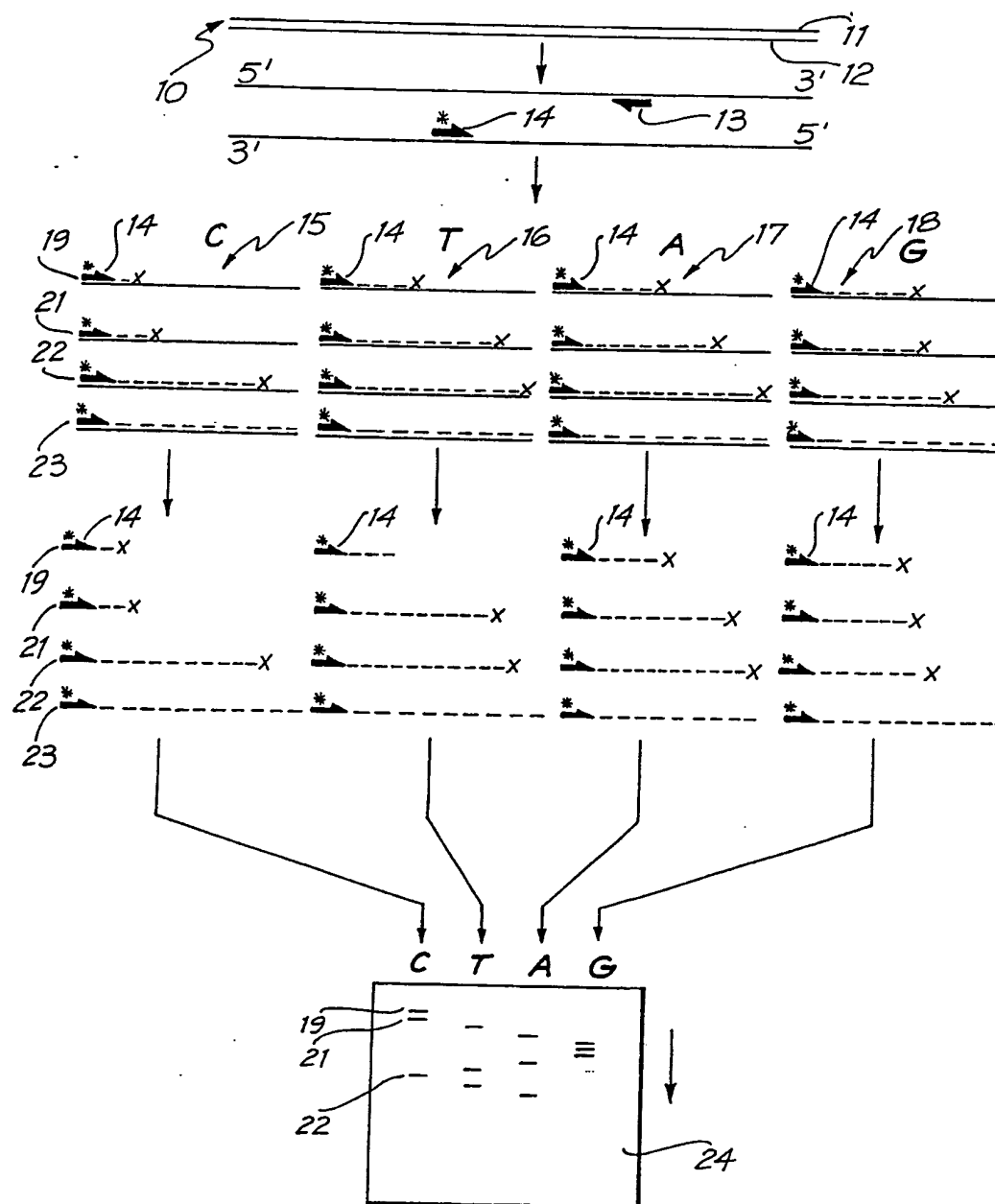


FIG. 1

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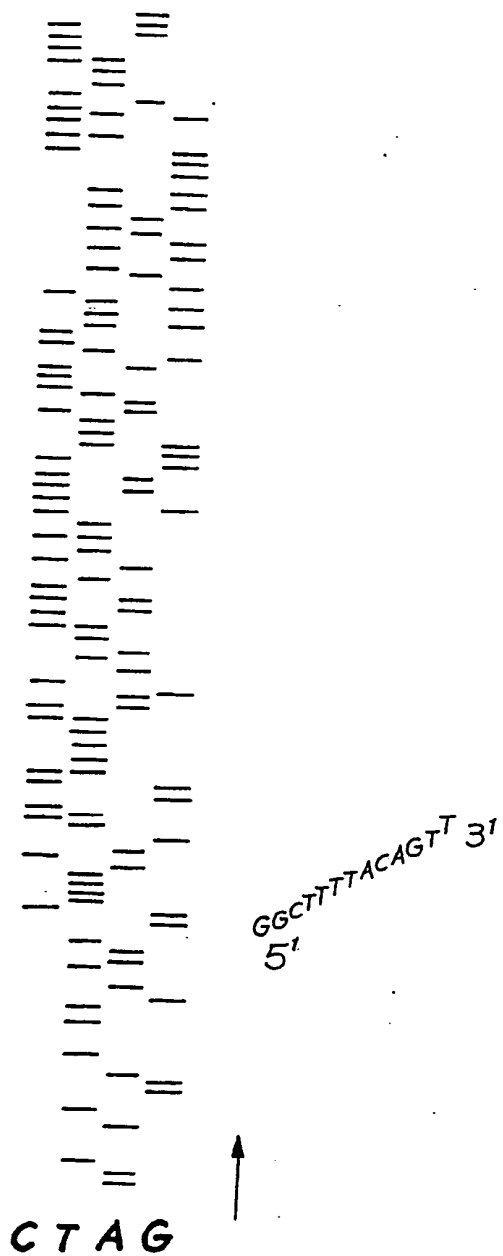


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00372

A. CLASSIFICATION OF SUBJECT MATTER
Int. CL⁵ C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT DATABASE; WPAT, BIOT-KEYWORDS POLYMERASE CHAIN REACTION, PCR, AMPLIFICATION, SEQUENCING.
CHEM AB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	AU 63411/90 (PROMEGA CORPORATION) 21 February 1991 (21.02.91) See page 9 lines 18-26 and Example 3	1-8
Y	AU 37986/89 (614245) (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 18 January 1990 (18.01.90) See whole document	1-8
Y	AU 45749/89 (623200) (ORION-YHTYMA OY) 7 June 1990 (07.06.90) See pages 6, 7, 9, 13, 14, examples and claims	1-8

☐ Further documents are listed
in the continuation of Box C.☐ See patent family annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

10 Nov 1992 (10.11.92)

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INTERNATIONAL SEARCH REPORT

International application No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
PY	AU 56754/90 (CONSEJO SUPERIOR INVESTIGACIONES CIENTIFICAS, UNITED STATES BIOCHEMICAL CORPORATION) 31 October 1991 (31.10.91)	1-8
Y	AU 33602/89 (CEMUBIOTEKNIK) 5 October 1989 (05.10.89) See whole document	1-8

**ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 92/00372**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
AU	63411/90	US	5108892	WO	9102090
AU	37986/89				
AU	45749/89	EP	371437	FI	895682
		JP	2219600	NO	894746
		US	5018336	IL	92474
				NZ	231576
AU	56754/90				
AU	33602/89	EP	406296	NO	904133
		WO	8909282	SE	8801070

END OF ANNEX